Dealing with DNA lesions: When one cell cycle is not enough

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Abstract

Subversion of genome integrity fuels cellular adaptation and is a prerequisite for organismal evolution, yet genomic lesions are also the harmful driving force of cancer and other age-related human diseases. Genome integrity maintenance is inherently linked to genome organization and nuclear architecture, which are substantially remodeled during the cell cycle. Here we discuss recent findings on how actively dividing cells cope with endogenous genomic lesions that occur frequently at repetitive, heterochromatic, and late replicating regions as byproducts of genome duplication. We discuss how such lesions, rather than being resolved immediately when they occur, are dealt with in subsequent cell cycle phases, and even after mitotic cell division, and how this in turn affects genome organization, stability, and function.

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Introduction

Among the main tasks of the cell nucleus are the functional organization and stabilization of the genome. This involves the spatial organization of DNA into nucleosomes, nucleosome clusters, chromatin loops, topologically associating domains (TADs), larger chromosome compartments, and chromosome territories [1,2]. But it also involves the constant remodeling of genome architecture to facilitate genome functions such as gene expression, DNA replication, cell division, and DNA repair. In this sense, the term *genome architecture* is misleading, as the genome is far from being static. Indeed, when genome organization is analyzed at single cell resolution, a continuous cell cycle–dependent reorganization of chromatin domains is revealed [3]. Genome duplication during S-phase of the cell cycle, chromosome condensation during mitotic cell division, and chromosome decondensation in early G1 all represent major, yet in their spatiotemporal regulation incompletely understood, genome remodeling events. Maintaining genome integrity throughout these processes is critical for cell function, and failure to do so is associated with accumulation of mutations, chromosomal aberrations, and cellular transformation leading to disease.

Genome integrity maintenance is the primary function of two interconnected cellular networks, the DNA damage response (DDR) [4,5] and the replication stress response (RSR) [6]. While the DDR takes care of chromosome breaks and other types of DNA lesions throughout the cell cycle, the RSR is primarily active in S-phase to deal with stalled replication forks and harmful replication intermediates. Although they are typically considered distinct cellular responses and often studied separately, emerging evidence suggests that certain replication intermediates, such as reversed replication forks, structurally resemble DNA lesions and that the DDR and the RSR employ a shared set of molecular players to maintain a stable genome [7]. Furthermore, both the DDR and the RSR trigger cell cycle checkpoints to slow down or halt cell cycle progression, and tight connections exist between the mechanisms involved in genome integrity maintenance and cell cycle control [8,9]. On the other hand, cell cycle checkpoints are not failsafe. Accumulating evidence suggests that replication intermediates and genomic lesions originating from endogenous replication stress can escape checkpoint surveillance and are transmitted to subsequent cell cycle phases and even to the next cell generation [10,11]. In this review we highlight and discuss regions in the genome that are particularly vulnerable to endogenous replication stress and thus inherently fragile, and provide an update on the emerging mechanisms used by cells to deal with this fragility in a manner that encompasses multiple successive cell cycle phases. As the genome undergoes

significant reorganization when cells cross the borders between cell cycle phases, for example, when they progress from S/G2 into mitosis and from mitosis into G1, we will put these mechanisms into the context of nuclear compartmentalization and discuss how this in turn may impact genome stability. Although dealing with DNA lesions across cell cycle boundaries is not limited to cancer cells, it may be particularly important as a source of cancer driving mutations, to shape cancer genome evolution, and to link deregulated genome integrity maintenance and genotoxic cancer therapies to innate immunity. Understanding the underlying principles and to which extent cancer cells rely on them may therefore offer new opportunities for therapeutic interventions.

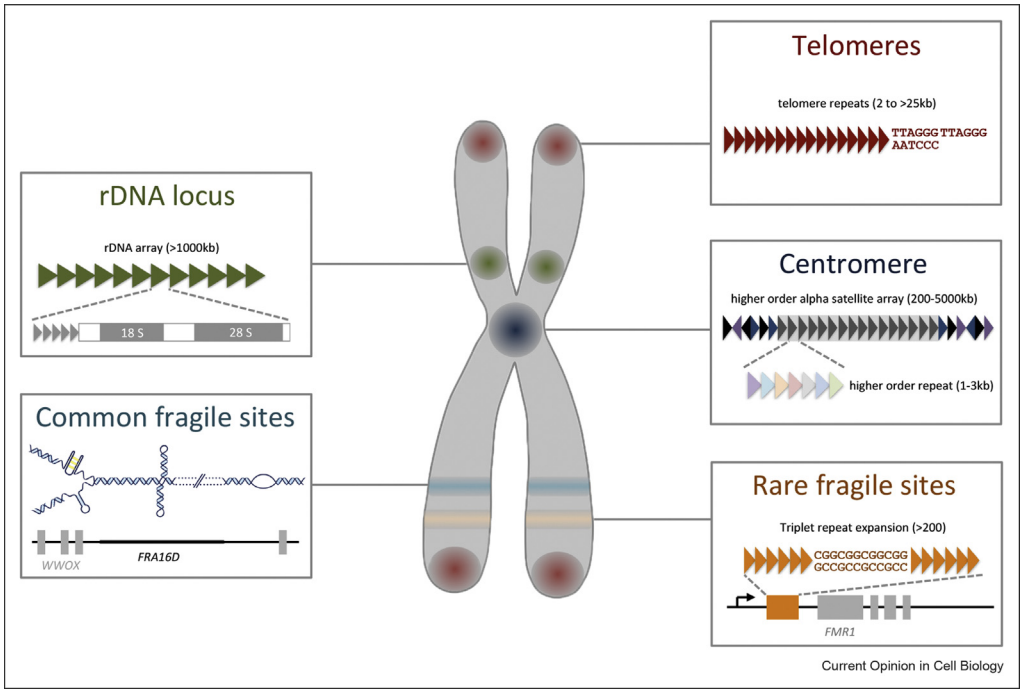
Endogenous replication stress and inherently fragile regions in the genome

Replication stress arises when replication fork progression is impaired [6]. This can occur in response to DNA lesions caused by exogenous DNA damaging agents, including many chemotherapeutic drugs, but it can also occur during normal S-phase progression in the absence of exogenous stress. Frequent replication fork slowing and transient pausing thus appear to be natural byproducts of genome duplication [7]. Among the sources for such endogenous replication stress are conflicts between the replication and transcription machineries, unusual DNA structures such as G-quadruplexes, bulky DNA-protein complexes, and difficult-to-replicate regions in the genome. The latter include chromosomal fragile regions, so-called common and rare fragile sites, as well as repetitive sequences at telomeres, centromeres, and ribosomal DNA (rDNA) (Figure 1). In addition to being inherently difficult to replicate, these regions are also replicated late in the cell cycle, and, as we will discuss in more detail below, their late replication timing significantly shortens the time window available in S-phase to complete their replication. This in turn primes them for replication carried on beyond S-phase, by mechanisms, which on one hand provide a safety net for completion of genome duplication but which on the other hand also pose a threat to genome integrity and render these regions particularly vulnerable.

Chromosomal fragile sites

Chromosomal fragile sites are hotspots for DNA breakage and aberrations such as deletions, duplications, and translocations in response to replication stress [12]. Based on their prevalence, fragile sites are typically classified as either rare or common (Figure 1). While rare fragile sites, such as the CGG triplet expansion in the *FMR1* gene underlying fragile X syndrome, are found only in a small percentage of the

Figure 1



Fragile genomic regions. Repetitive, heterochromatic, and late replicating regions in the genome are inherently fragile and frequently experience replication stress–associated damage. Fragile genomic regions include telomeres at chromosome ends and subtelomeric repeats, centromeres and pericentromeric repeats, rDNA repeats in the nucleolus, as well as common and rare fragile sites.

population, common fragile sites (CFSs), such as *FRA16D* and *FRA3B* with the associated tumor suppressor genes *WWOX* and *FHIT*, respectively, are present in the general population and frequently mutated in cancer [13]. CFSs are typically late replicating, origin-scarce regions. They are composed of AT-rich sequences, which can form secondary structures that stall replication and impair repair [14], and they are associated with very long genes at TAD boundaries that can take more than one cell cycle to be fully transcribed, inevitably evoking transcription-replication conflicts [15,16]. This combination of challenges impedes faithful and complete CFSs replication during S-phase, leading to under-replicated DNA and endangered CFSs stability [17]. Of note, transcription and replication timing are intertwined, and it was recently demonstrated that experimentally enhanced transcription, induced by a strong promoter, triggers a switch to earlier replication timing and alleviates fragility [18]. While this reinforces the notion that late replication of CFSs underlies their fragility, a distinct class of early replicating fragile sites in highly expressed gene clusters has also been identified [19,20••]. Their fragility is consistent with the recent finding that oncogene activation induces firing of normally suppressed replication origins within highly expressed genes, causing transcription-replication interference upon premature S-phase entry [21••]. Fragility thus not only occurs when time gets short at the end of S-phase but also at the transition from G1 to S-phase when high transcriptional activity collides with the rapid firing of origins to initiate replication.

Telomeres

Telomeres and the adjacent subtelomeric repeats are the regions that protect chromosome ends (Figure 1). In human cells, telomeres are composed of terminal TTAGGG repeat sequences, which can span several kilobases in length and which are characterized by constitutive heterochromatin. Despite their heterochromatic nature, the long noncoding RNA TERRA is transcribed from subtelomeric repeats and forms telomere-associated R-loops that can interfere with telomere replication and stability [22,23]. Secondary structures such as G-quadruplexes formed at telomeric G-rich repetitive sequences also challenge replication fork progression [24,25]. Replication stress at telomeres is further enhanced due to unidirectional replication downstream of the most distal origin, which means that irreversibly stalled replication forks cannot be rescued by dormant origin firing. Finally, the telomere-associated t-loop formed by the terminating single-stranded G-rich overhang and the tightly bound proteins of the protective shelterin complex challenge replisome progression and make telomere replication inherently difficult [26].

Telomere length maintenance, which is needed to overcome the end replication problem and achieve replicative immortality, depends on the reverse transcriptase telomerase, or in around 10–15% of cancers (including gliomas, sarcomas, and pancreatic neuroendocrine tumors), on alternative lengthening of telomeres (ALT) [24,27]. Telomere elongation by ALT relies on break-induced replication, a recombination-driven process that is initiated in the context of DNA replication but then continues as break-induced telomere synthesis beyond S-phase [28–33]. Break-induced replication additionally contributes to telomere fragility, and cancer cells using ALT for telomere maintenance show higher levels of telomere-associated replication stress compared to telomerase expressing non-ALT cancer cells [22,24,34••,35•].

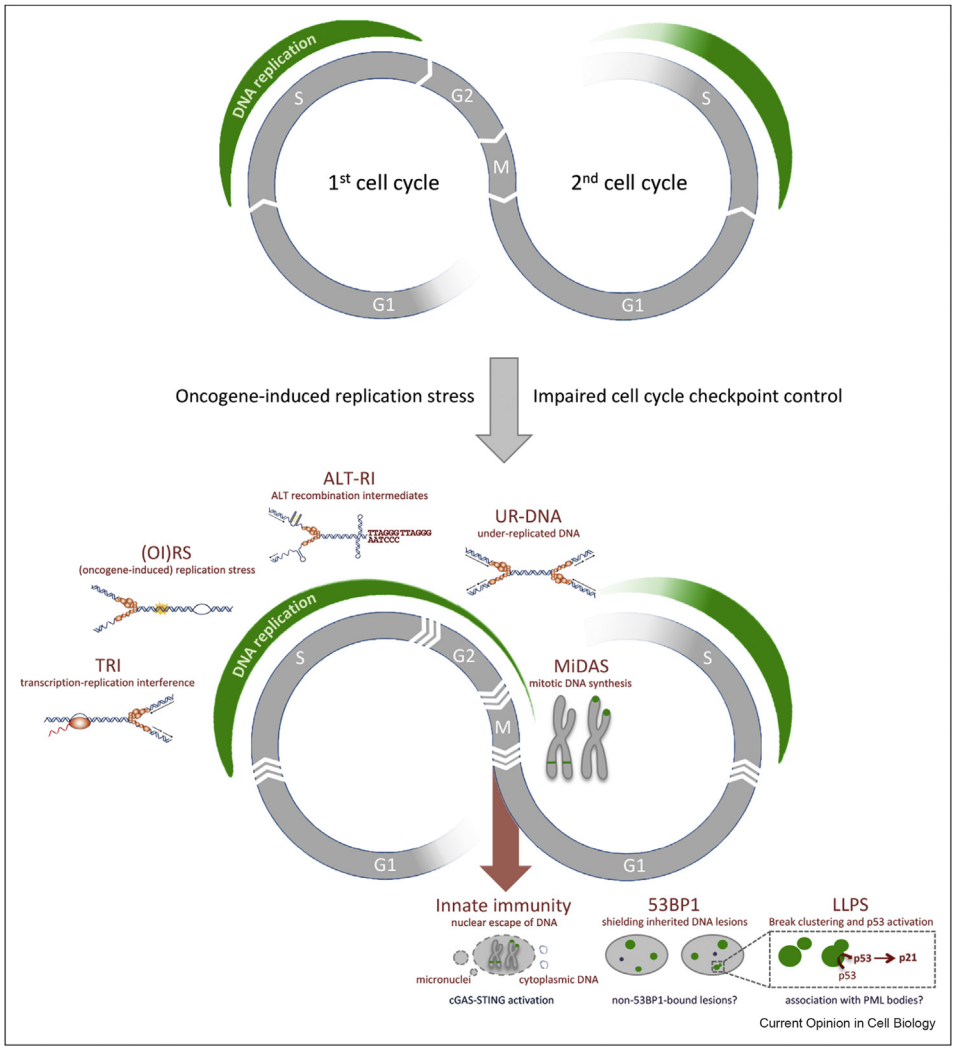
Centromeres

Centromeres are the assembly sites for the kinetochore, the protein complex that makes the connection between chromosomes and the spindle fibers, so that the chromatids can be pulled toward opposite poles during cell division (Figure 1). In human cells, centromeres are composed of a series of AT-rich head-to-tail tandem repeats named *alpha satellites*, which extend for several megabases [36]. Furthermore, the surrounding pericentromeric DNA is also organized in short tandem repeats. The repetitive nature of centromeric and pericentromeric sequences, the formation of DNA secondary structures, the heterochromatin environment and their generally late replication timing all contribute to centromere fragility [37]. Due to their central role for chromosome segregation, centromeric and pericentromeric replication stress, chromosome breaks and recombination are associated with chromosomal aberrations and translocations in cancer.

rDNA

Ribosomal DNA contains several hundred copies of repeated gene sequences (Figure 1). The ribosomal rRNAs, required for ribosome biosynthesis, are transcribed from these rDNA repeats, making them the most heavily transcribed regions in the genome. The human rDNA repeats encompass several genomic loci, with the 5 S rRNA gene repeats encoded by chromosome 1, and the 18 S, 5.8 S, and 28 S rRNA gene repeats distributed over chromosomes 13–15 and 21–22 [38]. These head-to-tail repeat clusters on multiple chromosomes come together in the nucleolus, a membraneless nuclear compartment. Due to an excess number of rDNA repeats, not all repeats are transcribed into rRNA. Silent rDNA repeats form tightly packed heterochromatin at the nucleolar periphery and, unlike actively transcribed repeats within the nucleolus, are replicated

Figure 2



Dealing with DNA lesions across cell cycle boundaries. While DNA replication is normally confined to the S-phase of the cell cycle, in response to endogenous and exogenous replication stress, e.g. upon oncogene activation or loss of tumor suppressor genes, replication and recombination intermediates are transmitted to later stages of the cell cycle. Late replicating regions, in which replication problems occur late in S-phase, are predestined for continuing replication in S/G2, and at under-replicated DNA replication continues even in mitosis. Mitotic DNA synthesis (MiDAS) is a conservative form of break-induced replication that works at common fragile sites and telomeres. Upon exit from mitosis, unresolved replication intermediates and chromosome breaks can give rise to micronuclei and free cytoplasmic DNA, such as extrachromosomal telomeric repeat DNA and C-circles, which in turn can activate the cGAS-STING pathway and trigger a type I interferon response. In the context of cancer treatment, chemo- and radiation therapy (including genotoxic cell cycle and DNA repair drugs such as ATR, WEE1, and PARP inhibitors) may thus synergize with immune response activating checkpoint inhibitors to maximize cancer cell death. In the newly forming main nucleus, 53BP1 condensates shield inherited DNA damage and are associated with clustering of difficult-to-repair lesions and a p53-p21-response. This extends G1 duration in response to replication stress during the previous cell cycle and determines whether and when cells commit to the next round of replication. In absence of such failsafe mechanisms (e.g. upon p53 or p21 loss), transgenerational lesion repair likely leads to a progressive accumulation of mutations and fuels cancer evolution.

late in S-phase. Rearrangements of rDNA repeats, rDNA repeat expansions and contractions, and rDNA-associated structural chromosomal aberrations are frequently found in cancer. The instability of rDNA in disease is likely driven by transcription-replication conflicts due to R-loops at transcribed rDNA repeats, and by replication impediments coming from silent, heterochromatic rDNA repeats and from their late replication timing.

Dealing with incompletely replicated DNA beyond S-phase

As mentioned above, inherently fragile regions may not only be susceptible to replication stress and DNA damage because of their difficult-to-replicate sequence but also because of their late replication timing. In particular in cancer cells, where cell cycle checkpoint control is loosened and premature cell cycle transitions occur, late replicating regions may continue replication

in G2 and even mitosis (Figure 2). Indeed, G2 could be viewed as an extended S-phase [39], as a time in the cell cycle when late replicating and under-replicated DNA is taken care of. Replication stress, in particular at origin scarce regions where fork stalling cannot be resolved by dormant origin firing, exacerbates the amount of incompletely replicated DNA. Likewise, shortening the S/G2 phase by overriding intrinsic checkpoint control and enforcing premature activation of mitotic processes, e.g. through inhibition of the ATR, CHK1, and WEE1 kinases [40–45], increases the fraction of the genome that has not been replicated fully. How do cells deal with such under-replicated DNA? Surprisingly, DNA replication does not seem to stop upon mitotic entry, as accumulating evidence has shown [46–52]. Mitotic DNA synthesis (MiDAS) occurs at CFSs and fragile telomeres and uses a form of break-induced replication. This conservative form of DNA synthesis depends on the replicative polymerase δ and its subunit POLD3, on the scaffolding factor SLX4, the MUS81-EME1 endonuclease, and on the RAD52 recombinase [53–58]. The switch from semi-conservative replication in S/G2 to MiDAS also seems to require prior replisome disassembly, which is mediated by the TRAIP ubiquitin ligase and CDC48/p97-dependent extraction of the replicative helicase from chromatin [59–62]. While the mechanism of MiDAS at CFSs and telomeres and the main players involved have started to emerge, to which extent MiDAS occurs at centromeres, rDNA repeats, and other fragile regions, and how different subpathways of MiDAS may deal with different fragile loci are currently not very well understood.

As genome duplication and cell division should be temporally separated in order to ensure that the complete genetic information is transmitted to the offspring, MiDAS may be the last resort to complete replication before chromosome segregation. Challenging this paradigm, it was recently shown in budding yeast that DNA synthesis is inhibited in metaphase due to high CDK activity, and allowed to resume during anaphase, late in mitosis when chromosomes separate [63••]. As mammalian MiDAS seems restricted to the time window from prophase to metaphase, whether post-metaphase DNA synthesis exists in human cells is currently not known.

What happens when MiDAS fails, or time is simply too short during mitosis to complete replication of under-replicated DNA coming from S/G2? Unresolved replication intermediates can lead to mitotic chromatin bridges, lagging chromosomes, nucleosome-free ultrafine anaphase bridges, and chromosome breaks [64–66]. These aberrations can give rise to 53BP1 nuclear bodies in G1 cells, which shield inherited genomic lesions from nucleolytic degradation and unscheduled repair reactions, and they can lead to broken DNA escaping from the newly forming nucleus to generate micronuclei and extrachromosomal DNA (Figure 2). Recent work has started to shed light on the consequences of such inherited genomic lesions, linking genome integrity maintenance to nuclear organization by liquid condensates and to cGAS-STING-mediated activation of innate immunity.

Consequences of replication stress–associated heritable DNA lesions

The time from nuclear envelope breakdown in prophase to its reformation in telophase poses a particular threat to genome integrity maintenance not only because of the massive chromatin rearrangements that happen during mitosis but also because the physical barrier that normally prevents leakage of DNA into the cytoplasm is gone. Although sophisticated mechanisms are in place to keep mitotic chromosomes spatially constrained and to separate nuclear from cytoplasmic components during nuclear reassembly [67••,68], broken pieces of chromosomes can escape from the newly forming nucleus to constitute micronuclei and cytoplasmic extrachromosomal DNA (Figure 2). Micronuclei have a fragile envelope that easily breaks down during interphase and they show asynchronous replication, which on one hand can lead to chromosome aberrations such as the complex rearrangements seen in chromothripsis, and on the other hand trigger, through cytoplasmic self-DNA sensing by the cGAS-STING pathway, an innate immune response [69••,70,71]. Telomere fragility and dysfunction during replicative crisis as well as extrachromosomal telomeric repeat DNA have also been associated with chromothripsis and cGAS-STING activation [72,73••,74•]. Nuclear escape of DNA late in mitosis as a consequence of replication stress and incomplete MiDAS at fragile genomic regions could therefore be a general mechanism that links deregulated or impaired genome integrity maintenance to genomic rearrangements and innate immune signaling (Figure 2).

The cGAS-STING pathway is also activated in cells that lost the tumor suppressors and homology-directed repair factors BRCA1 or BRCA2 [75–79]. Importantly, chemotherapeutic drugs that exacerbate replication stress and DNA damage, such as PARP and ATR inhibitors, result in more problems late in the cell cycle and during mitosis [41•,80•], and further stimulate the cGAS-STING-mediated type I interferon response, suggesting that such compounds can be exploited in certain contexts to boost cancer immunotherapies [81,82].

While mitotic exit bears the risk of losing damaged DNA from the newly forming nucleus, re-establishing the nuclear compartment also provides new opportunities to deal with inherited DNA lesions on correctly segregated chromosomes. The multivalent chromatin reader 53BP1 simultaneously senses replication status and presence of

DNA damage and through its oligomerization domain forms large compartments around genomic lesions in G1 cells (Figure 2). These protein condensates, which promote repair and stabilize the chromatin topology in the vicinity of DNA lesions [83••], show many features of liquid–liquid phase separation (LLPS), including concentration-dependent self-assembly and condensate fusions [84••,85••]. They are precluded from forming during mitosis, when the 53BP1 concentration is diluted after nuclear envelope breakdown and when high kinase activities, including CDK-dependent phosphorylations, prevent LLPS and 53BP1 assembly [86,87]. In G1, however, 53BP1 avidly assembles at inherited lesions and, via p53 and its downstream target p21, modulates G1 length and S-phase commitment [88–90]. Difficult-to-repair lesions inside such condensates are particularly prone to undergo clustering by compartment fusions [84••,91,92•], resulting in repair centers that facilitate repair but also bear the risk of promoting chromosomal translocations. The role of 53BP1 extends beyond G1, as it also impacts replication fork stability, replication timing of inherited lesions, and coordinates the hand-over to RAD52-mediated recombination later in the cell cycle [93•,94•].

While 53BP1 condensates are primarily associated with under-replicated DNA at CFSs, LLPS-mediated clustering has recently been described for inherently fragile telomeres in ALT-positive cancer cells [95•,96•]. ALT telomeres, mediated by the SUMO pathway, assemble into ALT-associated PML bodies (APBs), liquid-like condensates in which inter- and intrachromosomal break-induced replication occurs. As PML bodies are also recruited to persistent ionizing radiation-induced DNA lesions [97], compartmentalization into nuclear condensates may emerge as a common theme for a broader range of inherently fragile as well as difficult-to-repair genomic regions.

Conclusions

As opposed to the classic cell cycle arrest, repair, and restart model, work from recent years has started to support the notion that certain genomic lesions take more than one cell cycle phase and sometimes even more than one cell cycle to be repaired. Particularly replication stress-associated lesions and physiological replication intermediates, such as stretches of under-replicated DNA at late replicating regions in the genome, have a comparatively short time-window for repair in S-phase, and they depend on replication and repair events in late S/G2 and mitosis. This makes them especially fragile with consequences for genome stability and for the detection of DNA damage by the immune system.

Mutational cascades amplifying chromothripsis over successive cell cycles, and segregation of unrepaired genomic lesions over multiple cell generations drives evolution, subclonal heterogeneity, and adaptation of cancer genomes [98••,99••]. Furthermore, nonrandom segregation of DNA lesions in response to replication stress was recently shown to cause biased inheritance of newly replicated DNA by daughter cells [100••]. A better understanding of the underlying mechanisms and of the dynamic evolution of replication stress phenotypes may not only yield information on the origin and evolution of cancer but also hold promises for more targeted treatments. ATR inhibitors, for instance, enhance asymmetric distribution of replication stress-associated DNA damage [100••], and potentiate cGAS-STING-mediated interferon signaling [81]. In targeted cancer therapy, these two effects might synergize for desired outcomes.

While CFSs were the first fragile regions to be linked to 53BP1 nuclear bodies and MiDAS, telomeric MiDAS has recently received more attention. However, it is not entirely clear yet how ALT-negative and ALT-positive cancer cells differ in terms of MiDAS and replication stress-associated telomere fragility. The fragility of other genomic regions, including centromeres, pericentromeric repeats, and rDNA arrays, is even less well understood. New technologies that can map fragile regions and their replication genome-wide in specific cell cycle phases have recently been developed [101•,102•,103•], and they will likely shed new light on the mechanism of MiDAS-dependent recombination repair and also identify additional fragile regions. Applied to different cell types with different sets of activated oncogenes and inactivated tumor suppressors, a more comprehensive and condition-specific view of the fragile genome will be obtained. Complemented by time-resolved single cell experiments to investigate the fate of individual inherited DNA lesions for the next cell generations, we will understand better how cells deal with DNA damage when one cell cycle is not enough.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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